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# MOLECULAR PHYLOGENETICS OF SELECTED *ADIANTUM* SPECIES USING RBCL SEQUENCING STUDIES

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## ABSTRACT

Plants are used as a source of medicine in Traditional Medicinal Systems in India from time immemorial. The demand in medicinal plants leads to the adulteration in pharmaceutical industries. This study was performed to identify and analyze the phylogenetic relationship among four species of *Adiantum, A. latifolium, A. incisum, A. hispidulum* and *A.caudatum* using *rbcL* sequencing studies. The *rbcL* sequencing of the selected plants analysed with BLAST for similar sequence studies and the alignment was done with T-Coffee software. BLAST results indicated that the number of similar sequences present in the Genbank repository. The phylogenetic tree represented the degree of similarity andvariation with other species and other accessions of same species. Comparative analyses of these DNA barcode sequences revealed species-specific nucleotide sequences capable of discriminating the four *Adiantum* species. Phylogenetic analyses also revealed four distinct clusters showing homogeneous clades with high resolution at the species level. The result of the study was useful for the authentication ofselected *adiantaceae* members. The phylogenetic tree showed their relationship with other *adiantaceae* members.

**KEYWORDS:** DNA barcode, *Adiantum*species, Phylogenetic relationship, BLAST software.

## INTRODUCTION

Identification of plant species is of critical importance in conserving and utilising biodiversity, but this may be hindered by a lack of taxonomic expertise.[2] Other than identifying whole plants, it is also sometimes useful to be able to identify species from material such as roots, seeds, pollen or in mixtures of plants sampled from the air, soil or water, although this may be difficult or impossible using traditional morphological approaches.[1] A wide range of molecular techniques have been used to overcome this, but a growing desire for harmonization and increased efficiency has led to a global DNA barcoding initiative to standardise molecular identifications using internationally agreed protocols and regions of DNA.[5,7]

Reconstructing a tree of life by inferring evolutionary history is an important focus of evolutionary biology. Phylogenetic reconstructions also provide useful information for a range of scientific disciplines such as botany, zoology, phyto geography, archaeology and biological anthropology. Until the development of protein and DNA sequencing techniques in the 1960s

and 1970s, phylogenetic reconstructions were based on fossil records and comparative morphological /physiological analyses. Since then, progress in molecular phylogenetics has compensated for some of the shortcomings of phenotype-based comparisons. Comparisons at the molecular level increase the accuracy of phylogenetic inference because there is no environmental influence on DNA/peptide sequences and evaluation of sequence similarity is not subjective. While the number of morphological/physiological characters that are sufficiently conserved for phylogenetic inference is limited, molecular data provide a large number of data points and enable comparisons from diverse taxa. Over the last 20 years, developments in molecular phylogenetics have greatly contributed to our understanding of plant evolutionary relationships. Regions in the plant nuclear and organellar genomes that are optimal for phylogenetic inference have been determined and recent advances in DNA sequencing techniques have enabled comparisons at the whole genome level. Sequences from the nuclear and organellar genomes of thousands of plant species are readily available in public databases, enabling researchers

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without access to molecular biology tools to investigate phylogenetic relationships by sequence comparisons using the appropriate nucleotide substitution models and tree building algorithms

The majority preference of the CBOL Plant Working group was to recommend a core-barcode consisting of portions of two plastid coding regions, *rbcL+matK,* to be supplemented with additional markers as required.[1] The choice of *rbcL+matK* as a core barcode was based on the straightforward recovery of the rbcL region and the discriminatory power of the matK region. matK is one of the most rapidly evolving coding sections of the plastid genome.[6] and is perhaps the closest plant analogue to the CO1 animal barcode. Unfortunately, matK can be difficult to PCR amplify using existing primer sets – particularly in non-angiosperms. In contrast, the barcode region of *rbcL* is easy to amplify, sequence and align in most land plants and provides a useful backbone to the barcode dataset, despite it having only modest discriminatory power. Two-marker plastid barcodes gave better discrimination than single marker barcodes, but no other 2-marker or multi-marker plastid barcode gave appreciably greater species resolution than the *rbcL+matK* combination.[1] As both of these markers are coding regions, electronic translation of sequences from DNA to amino acids can be used to automate checks for editing/assembly errors, the presence of psuedogenes and correct sequence orientation. The coding and hence directly alignable nature of the data also facilitates character based analyses and comparative analyses of DNA barcode diversity among taxonomic groups and geographical regions.

In the present investigation the phylogenetic and taxonomical relationship of four *Adiantum* species were studied by *rbcl* sequence based DNA barcoding studies.

## MATERIALS AND METHODS

The four species of *Adiantum, A. latifolium, A. incisum, A. hispidulum* and *A. caudatum* were selected for the present study. The taxonomic features of the species have been confirmed with Flora of Presidency of Madras.[4] The healthy, young leaf samples of *Adiantaceae* members collected from the foot hills of Kothayar from November to February during the winter season. Specimens were collected with their fertile parts and herbarium specimens are prepared.

### DNA isolation and Agarose Gel Electrophoresis
About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. The DNA was isolated using NucleoSpin® Plant II kit (Macherey-Nagel). The isolated DNA was checked using 1% agarose gel. The banding pattern viewed under UV transilluminator. The PCR products checked in 1.2% agarose gel electrophoresis and viewed under transilluminator and photographed using gel documentation system. Forward primer ATGTCACCACAAACAGAGACTAAAGC and

reverse primer GGTAAAATCAAGTCCACCRCG were used for the study. PCR amplification profile for *rbcL* was 98ºC-30 sec, 98ºC-5 sec, 60ºC-10 sec, 72ºC-15 sec, 72ºC-60 sec and stored at 4ºC.

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems USA). The PCR profile for sequencing consists following cycles 96ºC for 2 minutes followed by 94ºC for 30 seconds, 50ºC for 40 seconds and 60ºC for 4 minutes for all primers for 30 cycles.

For post sequencing PCR clean up, master mix I of 10µl with 2µl of EDTA were prepared and mixed well. Master mix II with 2µl of 3M sodium acetate and 50µl of ethanol were prepared and mixed well. The products were incubated and centrifuged for 30 minutes and 100µl of 70% of ethanol with supernatant was added. The dried pellet was collected after centrifugation. The isolated result was sequenced through AB13500 DNA Analyzer (Applied Biosystems) and the quality of the sequence was analysed by sequence scanner software II. For sequence alignment and altering the obtained sequences were carried out by Geneious Pro v5.1.[9]
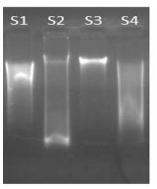
The species were identified from the representative DNA sequence of the DNA samples using the BOLD search engine. There are ten more similar sequences were identified and its sequences download in FASTA format using Basic Local Alignment Search Tool (BLAST) of GenBank. These sequences were aligned and compared with r*bcL* sequences generated for our plant samples using T- COFFEE software and MEGA4. Phylogenetic and molecular evolutionary analyses were carried out with MEGA.
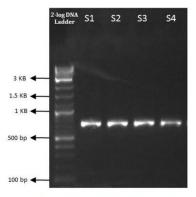
## RESULTS AND DISCUSSION

The discipline taxonomy (the science of naming and classifying organisms, the original bioinformatics and a basis for all biology) is fundamentally important in ensuring the quality of life of future human generation on the earth; yet over the past few decades, the teaching and research funding in taxonomy have declined because of its classical way of practice which lead the discipline many a times to a subject of opinion, and this ultimately gave birth to several problems and challenges, and therefore the taxonomist became an endangered race in the era of genomics. Now taxonomy suddenly became fashionable again due to revolutionary approaches in taxonomy called DNA barcoding (a novel technology to provide rapid, accurate, and automated species identifications using short orthologous DNA sequences). In DNA barcoding, complete data set can be obtained from a single specimen irrespective to morphological or life stage characters. The core idea of DNA barcoding is based on the fact that the highly conserved stretches of DNA, either coding or noncoding regions, vary at very minor degree during the evolution within the species. Sequences suggested to be useful in DNA barcoding

include cytoplasmic mitochondrial DNA (*e.g. cox*1) and chloroplast DNA (*e.g.* *rbcL*, *trnL-F*, *matK*, *ndhF*, and *atpBrbcL*), and nuclear DNA (ITS, and housekeeping genes (*e.g.* *gapdh*). The plant DNA barcoding is now transitioning the epitome of species identification; and thus, ultimately helping in the molecularization of taxonomy, a need of the hour. The 'DNA barcodes' show promise in providing a practical, standardized, species-level identification tool that can be used for biodiversity assessment, life history and ecological studies, forensic analysis, and many more.



**Figure 1: Genomic DNA, rbcL sequencing and Phylogenetic tree of Adiantum species.**

S1 – *Adiantum latifolium* **Lam.**
S2 – *Adiantum incisum* **Forssk.**
S3 – *Adiantum caudatum* **L.**
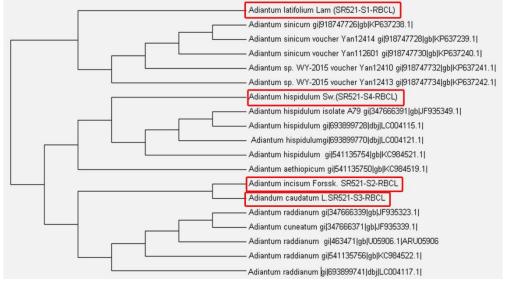S4 – *Adiantum hispidulum* **Sw.**



**Figure 2: Phylogenetic analysis of *rbcL* sequences of selected Adaintaceae members with their similar sequences through BLAST search.**

In the current research *rbcL* based DNA barcoding was studied for four *Adiantum species*. The genomic DNA of the *Adiantum* was isolated using standard protocol and purified for sequencing studies (figure 1). The purified product amplified with forward and reverse primers of universal *rbcL* primers. After that the sequencing was done with specific sequencer (figure 2). The resulting sequence was obtained from sequencer. The data was analyzed with T Coffee online server for sequence alignment and phylogenetic studies.

Before the data was analysed the similar sequence was obtained from BOLD[8] search engine and Nucleotide database, genbank of NCBI. The closely related five sequences were selected using NCBI- BLAST and the sequence was analysed with T- Coffee software.

Multiple sequence alignment was done with the help of T. Coffee software and the results were obtained.

Phylogenetic analysis was done with MEGA software. The Tree was constructed based on Neighbor Joining method. The trees showed the relationship between our

species and the related species. The phylogenetic tree was constructed with help of MEGA software. In DNA barcoding the sequences of the barcoding region are obtained from various individuals. The resulting sequence data are then used to construct a phylogenetic tree. In such a tree, similar, putatively related individuals are clustered together. The term 'DNA barcode' seems to imply that each species is characterized by a unique sequence, but there is of course considerable genetic variation within each species as well as between species. However, genetic distances between species are usually greater than those within species, so the phylogenetic tree is characterized by clusters of closely related individuals, and each cluster is assumed to represent a separate species.[3]

## CONCLUSION

DNA barcoding is of great utility to users of taxonomy. It provides more rapid progress then the traditional taxonomic work. DNA barcoding allows taxonomists to rapidly sort specimens by highlighting divergent taxa that may represent new species. DNA barcoding offers taxonomists the opportunity to greatly expand, and eventually complete, a global inventory of life's diversity. The advocates of DNA barcoding say that it has revitalizing biological collections and speed up species identification and inventories however the opponents argue that it will destroy traditional systematics and turn it into a service industry. Once fully developed, DNA barcoding will have the potential to completely revolutionize our knowledge of diversity of living organisms and our relationship to nature. By harnessing technological advances in electronics and genetics, DNA barcoding will help many people to quickly and cheaply recognize known species and retrieve information about them, and will speed discovery of thousands of species yet to be named. Barcoding has the potential to provide a vital new tool for appreciating and managing the Earth's immense biodiversity. DNA barcoding studies will helpful to know the evolutionary and taxonomic relationships of the plants.

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